PRINCIPLES OF COLUMN CHROMATOGRAPHY

Column chromatography follows the same principles as TLC, with the following differences:

- The stationary phase is contained inside a column, rather than applied as a coating on a plate. The column is commonly made of glass, but some are made of metal or other materials.
- The sample to be separated is loaded from the top. The eluting solvent, or mobile phase, is also added from the top.
- The solvent flows down the column by gravity, carrying with it the components of the sample. By the same principles that apply in TLC, the components travel at different rates effecting the separation.

Refer to figures 19.4 (p. 759) and 19.7 (p. 765) for diagrams of typical macroscale and microscale chromatographic columns. The macroscale column can be fitted with a valve to control the flow of solvent. Unfortunately, the microscale column is simply a Pasteur pipette that cannot be fitted with a convenient device to control flow. A small stopper or Teflon cap liner can be used to block the tip to interrupt solvent flow as needed.

It can be seen from fig. 19.4 that as in TLC, the less polar components travel down the column faster than the more polar ones. Once the less polar components are out the column, the polarity of the eluting solvent can be increased to speed up the rate of travel of the more polar components.

PREPARING A MICROSCALE COLUMN

Preparing a microscale column is very simple. Take a Pasteur pipette and place a small cotton plug at the part where it narrows into a capillary tube. Make sure the cotton plug is tight enough to hold the solid phase, but not too tight because that will result in a much slower rate of solvent flow. Also have a small rubber stopper or Teflon cap liner ready to control the solvent flow in the column.

Separately, make a slurry consisting of the solid support (alumina or silica gel) and the solvent to be used first. Secure the pipette vertically with a clamp, and place an empty beaker at the bottom to collect the solvent that goes through the column. Carefully pour small amounts of the slurry into the pipette with a dropper until the height of slurry that settles inside the pipette is about 1 – 1.5 inches.

During this time the solvent should be allowed to flow freely through the pipette and collected in the beaker at the bottom. However, the solvent level in the column should not be allowed to fall below the top of the slurry. If you see that this is about to happen, interrupt the solvent flow by blocking the bottom of the pipette and add more solvent or slurry, as it might be the case. See pictures on p. 764-765.

In other words, the slurry inside the column should never be allowed to dry out. If this happens it may create cracks and unevenness in the solid phase, which will decrease the efficiency of the separation.
RUNNING A CHROMATOGRAPHIC COLUMN

The sample to be separated is loaded in solution in a suitable solvent, preferably as concentrated as possible. Dilute samples tend to separate less efficiently.

1. When preparing the column as described above, also prepare a series of small test tubes for collecting the different fractions that will come out of the column as the separation proceeds. Also have masking tape and a marker to label the tubes, and of course a test tube rack.

2. After preparing the column as described above, allow the solvent to flow until its level comes very close to the top of the solid support, but not below it.

3. Load the sample solution in small amounts, allowing the solvent to flow after each load until it comes very close to the top of the solid support, but not below it. By doing this you never have large amounts of solvent present above the solid support.

4. After the sample is loaded, add the first eluting solvent (usually a low polarity one) in small amounts, just as you did with the sample. Once again, do not allow the solvent level to fall below the top of the solid support. By doing this you allow for complete adsorption of the sample onto the solid support.

5. Now you’re ready to proceed with the separation. Fill the top of the column with eluting solvent, and allow it to flow. If your sample is colored, as it is in experiment 15, you will see the sample move and separate into “bands” as the components of the mixture begin to separate.

6. As the solvent level reaches the top of the solid support, add more solvent to keep it from falling below the top of the solid.
7. As the solvent is flowing and you see the bands move down the column, be ready to **collect different bands into separate test tubes**. These collections are called **fractions**. If it is not clear where the band starts, play it safe by starting the collection before the band reaches the bottom of the pipette. It’s better to have an excess of solvent in your fraction than to lose material.

8. **The less polar components of the mixture will come out first.** The more polar components might lag far behind, moving very slowly. Once the less polar fractions come out, you can **gradually increase the polarity of the eluting solvent** to speed up the movement of the more polar bands. This is done by gradually increasing the percent of a more polar solvent from zero to whatever it takes to effect the separation. For example if your initial solvent was hexane, you can increase the polarity of the solvent by switching to 20% acetone / 80% hexane.

9. Continue this process until you’re satisfied that all the components of the mixture have come out with good separation. Keep in mind that **abrupt changes in solvent polarity might cause poor separations**. If there is a technique where **patience** is a premium asset, chromatography is definitely the one.

**ANALYZING THE FRACTIONS BY TLC**

The sample under analysis may or may not be colored. If it is colored, you can see the bands separate as they travel down the column. This enables you to more precisely collect the different fractions. However, it is always possible that each fraction may have more than one component if they all travel together due to similarities in their polarities and structures. It is good and common practice to further analyze each fraction by TLC. This process frequently reveals more than one component in fractions which may otherwise be taken as consisting of a single, pure substance.

In experiment 16 you will obtain at least two fractions from the chromatographic column, one yellow and one green. However, TLC analysis of each will further reveal the presence of several pigments in each fraction. Your job is to find out how many are there and what their $R_f$ values are. Your textbook lists the various pigments you are likely to see (p. 142), but this may vary from one type of spinach to another, and even with how fresh the leaf was at the time of the experiment.

If the sample under analysis is colorless, you cannot visually guide the fraction collecting process. In this case it is customary to collect fractions of equal size in different test tubes during the entire elution time. Each fraction is then analyzed by TLC. This will reveal first whether the fractions contain anything at all, and second whether they consist of a pure component or a mixture. All fractions containing the exact same components (according to their $R_f$ values and general appearance) are then combined. This process takes more time and more patience, but it has become largely automated in modern scientific and industrial laboratories where time savings translate into lower operational costs.
CRUSH LEAVES IN ACETONE

DECANT INTO C.T.

TRANSFER SUPERNATANT TO C.T. #2

2mL HEXANE
2mL H2O

 Shake
emulsion forms

emulsion breaks

2 LAYERS
TAKING THE DEEPEST COLORED ONE

Wet organic layer

Anhydrous Na2SO4

Dry organic layer (extract E)

Dry pigment

hexane solution

Dry fractions

RUN TLC IN
70% HEXANE
30% ACETONE

2 drops

Dry fractions

Alumina column chromatography

fraction